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DETECTION OF TRICLOSAN IN GEOGRAPHICALLY-DIVERSE INFLUENT WASTEWATER SAMPLES AND THE ISOLATION OF TRICLOSAN-RESISTANT BACTERIA

A Senior Honors Thesis in the Departments of Biology and Chemistry
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INTRODUCTION

Fate of Xenobiotics in the Environment

Significant concentrations of synthetic organic compounds have been detected in the aquatic environment.[1] Several classes of xenobiotics, including industrial chemicals, pesticides, fertilizers, surfactants, detergents, and pharmaceuticals are common constituents of water supplies.[2] Of particular interest are pharmaceutical compounds. Low concentrations of antimicrobials, antibiotics, chemotherapeutic drugs, and antiseptics have been identified in sewage, treated water, and rivers.[3] Despite their low concentrations, the risks associated with the presence of pharmaceutical compounds in water supplies have not yet been adequately assessed. Concerns have arisen regarding the potential health risks to humans, antibiotic resistance, and the ecological dangers associated with long-term exposure to low levels of pharmaceutical-tainted waters. Though pharmaceutical chemicals may enter potable water supplies, wastewater treatment procedures generally degrade the pharmaceutical substances into relatively innocuous metabolic conjugates.[4]

Pharmaceuticals are particularly distressing environmental pollutants because they are developed with the intention of performing a specific biological task and may be active at nanomolar concentrations. These compounds are often ionizable and lipophilic at some pH values so that they can penetrate cell membranes. In addition, they must be sufficiently stable chemically to produce a desired effect.[5] Thus, some pharmaceuticals

have the appropriate physicochemical properties that make them susceptible to bioaccumulation within aquatic and terrestrial environments.

Environmental effects stemming from the release of pharmaceutical compounds into water supplies have already been observed. Endocrine disrupting chemicals (EDCs) such as estradiol, polychlorinated biphenyls (PCBs), dioxins, furans, and synthetic steroids interrupt normal hormonal pathways in animals.[6] Wastewater treatment plants (WWTPs) receive synthetic and natural EDCs from domestic and industrial dischargers. Though WWTPs treat the influent waters, the efficiency of the treatment does not always completely transform or remove chemicals in the wastewater and the chemicals are ultimately discharged into surface waters. In Lake Mead (Las Vegas, NV), researchers have found estradiol in water reaching concentrations of 20 ng/l, a concentration that can cause some male fish to produce vitellogenin, an egg-synthesizing protein typically seen only in reproductive females.

In addition to EDCs, the widespread use of triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether or Irgasan® DP 300), a broad spectrum antimicrobial agent, has generated serious concerns regarding its fate and effects once released into the environment.[7]

Triclosan: Physical Properties

Triclosan (Figure 1) is a lipophilic, chlorinated biphenyl ether commonly utilized in toothpaste, deodorants, detergents, and in the treatment of some plastics and textiles.[8] Once triclosan is introduced into the environment after use and disposal, approximately

75 to 88% of the compound will biodegrade in aqueous streams.[9] It also adsorbs to activated sludge. Triclosan undergoes rapid photolysis when highly dilute aqueous solutions are exposed to sunlight; the aqueous photolysis half-life is less than one hour. In a report released by the United States Geological Survey, triclosan was detected at a median concentration of 0.14 μg/l in water samples from 139 stream sites across the United States.[10] It was the sixth most frequently detected compound, found in 57.6 percent of the sampled waters.

The toxicity of triclosan is pH-dependent; triclosan readily ionizes at a pH typically found in environmental water supplies. The ionized form of triclosan is generally less toxic than the neutral molecule, as the free acid has a greater propensity to cross the lipid membrane.[11] Triclosan is an acid that has a pK_a of 8.1 and at a standard environmental pH of 7.5 approximately 80% of triclosan exists in its neutral form.

Though it appears that triclosan is not toxic to mammals, triclosan has been shown to be acutely toxic to aquatic organisms such as *Oncorhynchus mykiss* (rainbow trout), *Daphnia magna* (water flea), and *Anabaena flos-aquae* (algae).[11, 12] Algae are particularly sensitive to triclosan. The *Anabaena flos-aquae* species of alga has an EC_{50} value of 2.8 µg/l. Similarly, the *Scenedesmus subspicatus* species has an EC_{50} value of 1.4 µg/l. Triclosan displays antimicrobial activity against both gram-positive and gram-negative bacteria, molds, and yeasts.[13] The minimum inhibitory concentration of triclosan for wild-type bacteria is reported as 0.1 µg/ml.[14]

Triclosan's Mechanism of Action

Triclosan inhibits the enoyl-acyl carrier protein reductase (FabI) involved in fatty acid biosynthesis.[15] The fatty acid synthase system in *Escherichia coli* is a type II or dissociated fatty acid synthase system, meaning that distinct genes encode each of the individual enzymes required for fatty acid biosynthesis.[16] The last elongation enzyme essential for the synthesis of lipids in *E. coli* is FabI, encoded by the *fabI* gene.

Kinetic and crystallographic studies have shown that triclosan binds tightly to FabI in the presence of a NAD^+ cofactor.[17] The phenolic ring of the triclosan molecule forms a stacking interaction with the nicotinamide moiety of the NAD^+. This interaction is a form of uncompetitive inhibition, indicating that triclosan binds to the enzyme-substrate complex. Increasing the concentration of FabI's natural substrate will not alleviate inhibition. X-ray studies on crystals containing NAD^+, triclosan, and *E. coli* enoyl-acyl carrier protein reductase show that triclosan is ionized to its phenolate state, mimicking the enolate moiety of FabI's natural substrate.[18]

Potential Bacterial Resistance to Triclosan

Bacterial resistance to triclosan has emerged as a public health concern. Recently, the Food and Drug Administration (FDA) advisory panel dismissed any linkages between the use of triclosan and antibiotic-resistant strains of bacteria, citing cases in which triclosan was used to control and reverse the outbreak of resistant bacteria in hospitals.[19] Some research has confirmed the FDA's findings. Because triclosan affects FabI in both the cytoplasmic membrane and intracellular targets, the cellular introduction of triclosan

culminates in cell death without mutating the bacterial genome.[20] This research corroborates the FDA's position that triclosan-resistant strains are unlikely to emerge.

Other research negates the FDA's assurance that triclosan-resistant strains are improbable. In laboratory settings, missense mutations have been observed in the *fabI* gene of *E. coli*, creating resistant strains. If these mutations do arise in the larger environment, the emergence of a resistance to triclosan may lower the efficacy of triclosan and similar antimicrobials that bind to the same site of the FabI enzyme.[21] Even if triclosan does not instigate genomic modifications, it will kill normal bacteria, creating an environment where resistant, naturally mutated bacteria are more likely to survive.[22]

Despite its specific targeting of the FabI enzyme, it was observed that wild-type *E. coli* required exposure to triclosan at a concentration of 150 μg/ml for two hours to achieve a 90% death rate.[23] Laboratory mutants more resistant to triclosan required two to four times this concentration for optimal efficacy. Furthermore, it was found that an average 5-second handwash with triclosan-enhanced detergent would not be sufficient to kill bacteria.

Several significant pathogenic bacteria lack the FabI enzyme, including the pseudomonads, and contain the FabI isoform FabK.[24] FabK, an enoyl acyl reductase similar in function to FabI, is intrinsically resistant to triclosan. Because triclosan is a broad spectrum antimicrobial, an alternative target for triclosan action must be present in species expressing the triclosan-resistant FabK enzyme.

In *Pseudomonas aeruginosa*, triclosan-resistance is conferred through the expression and selection of multidrug resistance (MDR) efflux pumps.[25] MDR efflux systems are composed of nonspecific protein translocases embedded in the plasma membrane of a bacterial cell.[26] Due to their lack of specificity, MDR pumps may facilitate the export of structurally unrelated drugs. The *P. aeruginosa* genome contains structural genes for at least twelve different MDR systems. Triclosan is one of the many substrates actively exported from *P. aeruginosa* cells. Thus, *P. aeruginosa* MDR systems are capable of selecting bacterial cells resistant to triclosan exposure.

Triclosan's ability to select for MDR systems in *P. aeruginosa* introduces a multitude of clinical and therapeutic concerns. Antibiotic-resistant strains of *P. aeruginosa* are already the leading cause of death in hospital acquired infections.[27] Similarly, most cystic fibrosis deaths are the result of chronic *P. aeruginosa* infections that have developed high-level resistance to antipseudomonad therapies.[28]

In addition to its inhibitory effects against the FabI enzyme, triclosan appears to be an inhibitor of the FabI homologue in mycobacteria, InhA. Structural studies have confirmed that pharmaceuticals used to treat tuberculosis such as ethionamide and isoniazid target InhA.[29] Isoniazid is a prodrug, requiring treatment with the catalase KatG prior to binding to the InhA active site. Ethionamide appears to target InhA in the same manner as isoniazid. These two drugs selectively bind to the InhA enzyme, halting production of mycolic acids that are critical components of the bacterial cell wall. The

FabI enzyme of *E. coli* and the InhA enzyme of mycobacteria share key binding residues with an overall 40% homology.

Because mycobacteria have a single binding site on the InhA enzyme for both triclosan and isoniazid, concerns have arisen regarding potential cross-resistance between the two compounds. *Mycobacterium tuberculosis*, the microorganism implicated in tuberculosis infections, kills 3 million people each year.[30] The spread of tuberculosis is enhanced by the development of mycobacterial strains resistant to front-line antitubercular drugs. Many of the antitubercular pharmaceuticals, such as isoniazid, rifampin, and ethionamide, target the InhA enzyme, found exclusively in mycobacteria. Thus, the development of mycobacterial resistance to triclosan may potentially confer resistance to antituberculosis drugs. Furthermore, the development and testing of novel antituberculosis pharmaceuticals takes a minimum of three years, while mycobacteria become resistant to these pharmaceuticals in an average time frame of three months.[31]

Degradation of Triclosan by Microorganisms

While genetic mutations to the bacterial genome are a concern, certain microorganisms ubiquitous in the natural environment are capable of chemically modifying and degrading triclosan through biocatalytic transformation. The white rot fungi, *Trametes versicolor* and *Pycnoporus cinnabarinus*, appear to utilize glucosylating and xylosylating enzymes in the addition of carbohydrate moieties to triclosan.[32] Through the addition of various sugar groups to the triclosan molecule, triclosan is effectively inactivated and the fungi are protected against the intended effects of the xenobiotic. The white rot fungi also

convert triclosan into 2,4-dichlorophenol in small quantities through cleavage of the ether bond between aromatic rings.

In a bacterial consortium, some microorganisms are capable of utilizing triclosan as the sole carbon and energy source.[33] Triclosan metabolism is immediate in the presence of these microorganisms, suggesting that the metabolic process is a detoxification mechanism adopted by certain bacteria. In addition, the process is most likely the result of cometabolism, requiring auxiliary catabolic enzymes from different bacterial strains. No triclosan metabolites produced by triclosan-degrading bacteria have been identified, as opposed to the glycosylated metabolites identified in fungal triclosan degradation.

Two triclosan-resistant microorganisms found in soil samples, *Achromobacter xylosoxidans* (formerly *Alcaligenes xylosoxidans*) and *Pseudomonas putida*, have been identified as bacteria capable of utilizing triclosan as their sole carbon source.[34] Both strains of bacteria are responsible for nosocomial infections and *Achromobacter xylosoxidans* is the cause of bacteremia in immunocompromised hospital patients.[35] The use of triclosan to eradicate such organisms in a hospital-setting may be ineffective.

Concerns Regarding the Release of Triclosan into the Environment

Because triclosan is a chlorinated phenolic compound, it is capable of forming dioxins under certain conditions. The dioxins are a family of 210 nonbiodegradable compounds.[36] Produced in the natural environment and by industrial processes, dioxins

are capable of accumulating in adipose tissue, increasing the risk of tumors and potentially instigating other deleterious health effects.

Predioxins, like triclosan, have been shown to undergo thermal and photochemical ring closure to form polychlorinated dibenzo-*p*-dioxins (PCDDs) attributed to WWTP chlorination methods.[37] Chlorination is used extensively in WWTPs to disinfect and deodorize effluents prior to their discharge, particularly when the water may subsequently be used for recreational purposes or as a source of drinking water.

It has also been observed that triclosan will form PCDDs and other dioxins following pyrolysis and intense ultraviolet radiation.[38] Thus, it may be possible for triclosan released in the environment to form dioxins under extreme conditions. Incineration of sewage sludge is potentially hazardous as well; chlorinated 2-phenoxyphenols and other predioxins can undergo ring closure at elevated temperatures.[39] The burning of sludge may produce extremely toxic chlorinated dibenzo-*p*-dioxins.

Purpose and Rationale of Wastewater Research

Due to the concerns regarding the fate and effect of triclosan in the environment, the triclosan concentration in geographically-diverse influent wastewater samples was assessed. Influent wastewater from multiple treatment plants in Virginia was sampled to determine the triclosan concentration and the level of concentration variability among the different treatment facilities. In addition, triclosan-resistant microorganisms from

wastewater were isolated and identified. The susceptibility of the triclosan-resistant bacteria to other antibiotics was also investigated.

MATERIALS AND METHODS

QUANTIFICATION OF TRICLOSAN CONCENTRATIONS IN WASTEWATER

Wastewater Collection. Primary influent wastewater samples were collected from the Rivanna Wastewater Treatment Plant at Moores Creek in Charlottesville, VA; the Lexington Wastewater Plant in Lexington, VA; the Lynchburg Regional Wastewater Treatment Facility in Lynchburg, VA; and the Roanoke City Wastewater Treatment Plant in Roanoke, VA. The samples were transported back to the laboratory in a cooler with ice packs and stored at 4°C for no longer than 48 hours prior to extraction.

Organic Extraction of Wastewater Samples. Five milliliters of HPLC-grade acetonitrile (Mallinckrodt Baker, Paris, KY) and 2 ml of concentrated sulfuric acid (VWR Scientific Products, West Chester, PA) were added to 1-liter wastewater samples. The pH of this mixture was approximately 2. The samples were filtered under a vacuum using 9.0-cm Whatman filters. After filtration, two 50-ml fractions of pesticide-grade hexane (95% n-hexanes; Mallinckrodt Baker, Paris, KY) were used to extract the organic components from the wastewater. The hexane layer was evaporated to dryness using a rotary evaporator and resuspended in approximately 4 ml of acetonitrile. The sample was then filtered through a 0.45-μ Acrodisc filter and stored in 1.5-ml HPLC sample vials.

HPLC Analysis. An Agilent 1100 Series HPLC was used for analysis of the samples. A 250 mm x 4.60 mm x 5 µm Phenomenex Luna C18 column was used to separate triclosan from other extracted compounds and a 30 mm x 4.60 mm x 5 µm Phenomenex Luna C18 guard column was used to protect the analytical column. The samples were eluted using an isocratic method with a mobile phase composed of HPLC-grade acetonitrile and 0.1%

(v/v) acetic acid in HPLC-grade water (EM Science, Darmstadt, Germany). The mobile phase was held at 60:40 acetonitrile:water for 40.0 min. After the sample had eluted from the column, the mobile phase was adjusted to 100 percent acetonitrile to elute any remaining compounds from the analytical column prior to analysis of the next sample. Elution was monitored at 230 nm using a diode array detector. A flow rate of 1.0 ml/min with an injection volume of 100 μl was used. Samples were analyzed three times and a mean solution concentration and standard deviation were calculated. Triclosan peaks from the sample chromatograms were identified through comparison to the retention times of triclosan reference standards (Ciba Specialty Chemicals, High Point, NC).

Quantification of Triclosan in Wastewater Samples. Triclosan standard samples at varying concentrations (20, 50, 100, 300, 500 μg/l) were analyzed through HPLC. Area counts for each standard sample were recorded and plotted against the respective concentration. A standard curve and equation were generated. The area counts for the triclosan-peaks from the wastewater samples were determined using Agilent ChemStation software. Triclosan concentrations in the wastewater samples were derived using the linear equation from the standard curve.

Confirmation of Triclosan in Wastewater. A Hewlett-Packard 5890 Series II Plus Gas Chromatograph and Hewlett-Packard 5972 Series Mass Selective Detector were used to confirm the presence of triclosan in the wastewater samples. The GC-MS was equipped with a nonpolar Supelco capillary column (30 m x 0.25 mm x 1.0 μm). The injector temperature was 300°C and the detector temperature was maintained at 280°C. The temperature program used was 40°C (1 min), 10.0°C/min, 70°C (5 min), 20°C/min, 310°C (15 min). A 974 mg/l triclosan standard, an extracted wastewater sample from the

Lynchburg Regional Wastewater Treatment Facility spiked with triclosan, and an extracted sample from the same plant with no spike were injected into the GC/MS.

ISOLATION AND IDENTIFICATION OF TRICLOSAN-RESISTANT MICROORGANISMS FROM WASTEWATER

Isolation of Triclosan-Resistant Microorganisms. Influent wastewater was collected from the Rivanna Wastewater Treatment Plant at Moores Creek in Charlottesville, VA. The sample was aerated and enriched with nutrient broth prior to use. The wastewater was diluted in PBS buffer (1:100) and a 150-μl sample of the diluted wastewater was spread onto R2A agar plates enriched with concentrations of 10, 15, and 20 mg/l of triclosan. Due to triclosan's low solubility in water, acetone was used as the carrier solvent. The control R2A agar plates with no triclosan in the agar contained a volume of acetone equivalent to the amount used as the carrier solvent in the triclosan test plates. The plates were incubated at 37°C for 24 hours and individual colonies growing on these plates were streaked onto triclosan-enriched plates at concentrations of 20, 50, and 100 mg/l. The bacteria were grown at 37°C for 24 hours. Isolated bacteria were assigned strain numbers.

Resistance of the Triclosan-Resistant Strains to Antibiotics. Triclosan-resistant strains were grown in nutrient broth and lawned onto R2A agar plates. Rifampin (5 μg; Becton, Dickinson and Co., Le Pont de Claix, France), ciprofloxacin (5 μg; Becton, Dickinson and Co., Le Pont de Claix, France), penicillin (10 μg; Becton, Dickinson and Co., Le Pont de Claix, France), ampicillin (2 μg; Becton, Dickinson and Co., Le Pont de

Claix, France), and streptomycin (10 μg; Becton, Dickinson and Co., Le Pont de Claix, France) antibiotic disks were aseptically placed onto each plate. The plates were incubated at 37°C for 24 hours and the zones of inhibition were measured.

Identification of Triclosan-Resistant Organisms. Triclosan-resistant strains were stained to determine whether the bacteria were Gram-positive or Gram-negative. Using the Biolog (BIOLOG, Inc., Hayward, CA) microbial identification system and GN2 MicroPlates^{TM}, the identities of the triclosan-resistant strains were determined by comparing their biochemical profiles with the profiles provided by the Biolog database. The Biolog system consists of 96-well microtiter plates containing 95 separate biochemical substrates.

RESULTS

QUANTIFICATION OF TRICLOSAN CONCENTRATIONS IN WASTEWATER

The concentrations of triclosan in primary influent wastewaters were determined through HPLC analysis of the extracted wastewater samples. In order to determine the retention time of triclosan, a standard triclosan solution dissolved in 60:40 acetonitrile:water was injected into the HPLC. The standard triclosan sample had a retention time of 25.9 minutes (Figure 2). In addition, a standard curve was plotted using area counts collected from chromatograms of known triclosan concentration injections. The R^2 value of the standard curve was 0.99. Chromatograms for each sample were generated (Figures 3-6). Using the standard curve, the concentrations of triclosan in the four wastewater samples were quantified (Table 1). The presence of triclosan in the wastewater sample was confirmed using GC-MS and the standard triclosan sample peak was observed at 19.33 minutes (Figure 7). Similarly, the non-spiked wastewater sample had a triclosan-peak at 19.28 minutes (Figure 8). The mass spectra from each sample were evaluated (Figure 9).

ISOLATION AND IDENTIFICATION OF TRICLOSAN-RESISTANT MICROORGANISMS FROM WASTEWATER

Diverse colony morphology was observed on the control plates. A small number of bacterial colonies were also observed growing on the 10, 15, and 20 mg/l triclosan-enriched plates. These colonies were isolated and subcultured onto 20, 50, and 100 mg/l triclosan-enriched plates. At these respective concentrations, the colonies grew well. No

differences were observed in the rate or amount of growth at the varying triclosan concentrations.

Varying levels of resistance to rifampin, ciprofloxacin, streptomycin, penicillin, and ampicillin were observed, though all strains showed either complete or intermediate resistance to rifampin (Table 2). One strain was resistant to ciprofloxacin, several strains showed an intermediate resistance to this antibiotic, and most were susceptible to the effects of the compound.

Gram-staining of the triclosan-resistant colonies showed that all of the strains are gram-negative (Figure 10). Furthermore, all of the strains have a general bacillus morphology. The strains were identified using the Biolog protocol (Table 2).

DISCUSSION

QUANTIFICATION OF TRICLOSAN CONCENTRATIONS IN WASTEWATER

Significant concentrations of triclosan were found in influent wastewaters, indicative of triclosan's widespread use as an antimicrobial in soaps, detergents, toothpastes, and other commercial projects. The greatest concentration of triclosan was found at the Lynchburg Regional Wastewater Treatment Facility (Lynchburg, VA) with a mean concentration of 15.5 μg/l. Lesser, yet appreciable, concentrations were found at the other treatment facilities.

Triclosan concentrations varied among the tested influent samples collected from the various wastewater treatment facilities. The source of the wide range of triclosan concentrations is unknown, though an interesting point of speculation. It was initially hypothesized that triclosan concentrations would be most significant at the Rivanna Wastewater Treatment Plant in Charlottesville, VA, as it is the recipient of wastewater from both the University of Virginia and its associated medical facilities. However, Charlottesville wastewater had a mean triclosan concentration of 3.77 μg/l, much less than that quantified from Lynchburg. The Lynchburg Regional Wastewater Treatment Facility receives much of its water from neighboring factories and industries, in addition to the wastewaters from surrounding residential areas.

The triclosan peak on the HPLC chromatogram for the Lynchburg site is not symmetrical. In addition, the peak has a shoulder, indicating that the triclosan peak may

be superimposed over a second peak. This would increase the area of the peak and increase the estimated concentration of triclosan in the Lynchburg wastewater. The area count of the postulated triclosan portion of peak is approximately one-fourth of the entire peak area. The concentration of triclosan in the wastewater would then decrease to approximately 4 μg/l.

The ultimate effect of triclosan in the environment is unknown. However, triclosan has been shown to be toxic to certain aquatic organisms. The unicellular alga *Anabaena flos-aquae* has a 96-h EC50 value of 1.6 μg/l; algae exposed to larger triclosan concentrations showed almost no growth.[11] The triclosan concentrations in all examined influent wastewater samples surpassed the EC50 value for unicellular algae, and the 15.5 μg/l triclosan concentration in the influent wastewater collected from the Lynchburg Regional Wastewater Treatment Facility was almost ten times as great as the observed EC50 value for unicellular algae.

Triclosan exists primarily as a neutral compound at environmental pH. Because neutral molecules are generally more toxic than their ionized counterparts, significant triclosan concentrations in influent wastewater samples may be cytotoxic to aquatic organisms. Triclosan in its ionized state may be less likely to cross a lipid membrane, potentially lessening its ability to target intracellular enzymes and macromolecules.

No data implicate triclosan as a human toxicant. Nonetheless, high concentrations of triclosan have been found in human milk samples at a concentration as high as 300

μg/kg.[13] Similarly, trace amounts of triclosan have been identified in human blood plasma samples.[41] Due to the structure of the triclosan molecule and the promiscuous nature of the estrogen receptor, it is possible that triclosan may be able to occupy and potentially trigger alterations in endocrine activity, though there is currently no data implicating triclosan in endocrine disruption.

Bacterial resistance to triclosan is also emerging as a widespread environmental concern. While triclosan is a broad-spectrum antimicrobial, it specifically inhibits an enzyme involved in fatty acid biosynthesis. Resistance to triclosan introduces a concern regarding resistance to other therapeutic agents that affect the same enzyme, including frontline antituberculosis and antimalarial pharmaceuticals. It is conceivable that significant concentrations of triclosan, such as those observed in the influent wastewater, could provide a selective advantage for bacteria that display greater resistance to the inhibitory effects of triclosan.[40]

ISOLATION AND IDENTIFICATION OF TRICLOSAN-RESISTANT MICROORGANISMS FROM WASTEWATER

Several strains of triclosan-resistant bacteria were isolated from influent wastewater. These isolated strains were capable of growing on triclosan-enriched media at concentrations of 100 mg/l despite triclosan's broad spectrum mechanism of action. In

addition to triclosan resistance, many of the isolated bacteria were resistant to an assortment of frontline antibiotics.

Isolated triclosan-resistant bacteria include *Aeromonas caviae DNA group 4, Aeromonas media DNA group 5B, Ralstonia eutropha, Pseudomonas nitroreducens/azelaica,* and *Achromobacter xylosoxidans.* Although all of these bacteria show high-level resistance to triclosan, they vary in their susceptibility to other antibiotics. Bacteria belonging to the same species generally show similar patterns of resistance. For example, all bacteria identified as *Achromobacter xylosoxidans* are resistant to rifampin, penicillin, and ampicillin, while most show intermediate resistance to ciprofloxacin.

The lack of uniformity of the antibiotic resistance among the triclosan-resistant species is not surprising due to the nature of antibiotic resistance. Resistance is conferred when the target enzyme or organelle adopts a mutation that enables it to survive when exposed to the antibiotic. The genes encoding for resistance are often found on R plasmids separate from the linear bacterial chromosome.[27] Such plasmids are easily and randomly exchanged between bacteria through conjugation, transduction, and transformation. The variety of susceptibilities among the isolated bacteria found in this study are suggestive of the random adoption of antibiotic-resistance genes.

The mechanisms that these wastewater bacteria use to survive at significant concentrations of triclosan and other antibiotics is unknown, though previous studies suggest that some of the bacteria degrade and effectively inactive triclosan. The soil

bacteria *Pseudomonas putida* and *Achromobacter xylosoxidans* (formerly *Alcaligenes xylosoxidans*) are known to degrade triclosan. In nutrient-poor media, these microorganisms may utilize triclosan as a source of carbon, although degradation may also be a survival mechanism that the bacteria can utilize against the adverse effects of triclosan. Multiple triclosan-resistant strains of *Achromobacter xylosoxidans* were found in the influent wastewater samples. While it is thought that these strains utilize a similar degradation pathway as the soil microorganisms, research is currently being conducted to confirm this hypothesis.

Achromobacter xylosoxidans, one of the triclosan-resistant families found in wastewater, is a common opportunistic pathogen considered to be the etiological agent in neonatal nosocomial infections and infections in immunocompromised individuals.[42] Although it is not a major human pathogen and community-acquired *Achromobacter* infections are uncommon, it is uniformly resistant to penicillin, ampicillin, and rifampin with intermediate susceptibility to chloramphenicols and tetracyclines.[43] Additional resistance to triclosan could be problematic in a hospital setting as triclosan is a common disinfectant used to eradicate common microorganisms such as *Achromobacter xylosoxidans*.

Only one *Pseudomonas* strain was identified in the wastewater sample, *Pseudomonas nitroreducens/azelaica*. This was a surprising result, as pseudomonads are common wastewater-dwellers and have a natural resistance to triclosan due to the presence of the FabK enzyme in many pseudomonads. Another possible mechanism of triclosan-

resistance in the *Pseudomonas* genus is through the utilization of a biphenyl 2,3-dioxygenase enzyme.[44] Bacteria that have been isolated from areas with high concentrations of polychlorinated biphenyls are capable of oxidizing chlorinated biphenyl cogeners. Oxidation of the triclosan molecule could potentially affect its ability to bind to the enzymatic active site. Similarly, certain *Pseudomonas* strains isolated from soil samples are capable of degrading 3- and 4-carboxybiphenyl ether through dioxygenolytic cleavage of the ether bond, further suggesting that an oxygenase may degrade triclosan into an inactive conjugate.[45]

Triclosan-resistant *Aeromonas* strains were also prevalent in influent wastewater. Over the past several years, antibiotic-resistant *Aeromonas* species have emerged as opportunistic pathogens implicated in the skin infections, gastroenteritis, and in diseases afflicting immunocompromised hospital patients.[46] *Aeromonas caviae DNA group 4* showed complete or intermediate resistance to rifampin, penicillin, ampicillin, and streptomycin. Conversely, *Aeromonas media DNA group 5B* was susceptible to both ampicillin and ciprofloxacin.

Although none of the isolated triclosan-resistant bacteria are considered major human pathogens, their combined resistance to triclosan and frontline antibiotics is a concern, as these bacteria are ubiquitous environmental organisms. These microorganisms appear to have several mechanisms available to them with which they can inactivate or resist triclosan, including spontaneous genetic mutations, enzymatic degradation of triclosan, or chemical modification of the triclosan molecule. Regardless of the method that the

triclosan-resistant bacteria utilize to survive, triclosan resistance should be addressed as a public health concern.

In conclusion, significant concentrations of triclosan were detected in influent wastewaters across Virginia. Although the ultimate effect of triclosan in influent wastewater is unknown, the amounts of triclosan found in the wastewater samples are large enough to be toxic to certain aquatic organisms. Triclosan-resistant bacteria were also found to inhabit influent wastewater. It is likely that these bacteria degrade triclosan to inactivate the toxic effects of the molecule. The isolated triclosan-resistant bacteria were also found to be resistant to a host of common antibiotics, including ampicillin, penicillin, streptomycin, ciprofloxacin, and rifampin.

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Table 1. Triclosan data collected from HPLC of extracted primary influent samples

Wastewater treatment facility	Mean triclosan retention time (min)	Mean triclosan concentration (μg/l)	Standard deviation of mean concentration (μg/l)
Rivanna Wastewater Treatment Plant – Moores Creek (Charlottesville, VA)	25.47	3.8	1.6
Lexington Wastewater Plant (Lexington, VA)	25.49	8.1	1.6
Lynchburg Regional Wastewater Treatment Facility (Lynchburg, VA)	25.64	15.5	1.7
Roanoke City Wastewater Treatment Plant (Roanoke, VA)	25.64	2.2	0.7

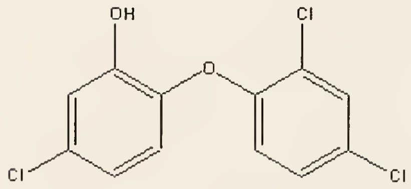
Table 2. Measured zones of inhibition (mm) of bacteria to antimicrobials.*

Strain Number	Identification†	Rifampin	Ciprofloxacin	Penicillin	Ampicillin	Streptomycin
10.1.1	*Aeromonas caviae DNA group 4*	R	25 (S)	R	14 (R)	11 (R)
10.1.2	*Aeromonas caviae DNA group 4*	12 (R)	24 (S)	17 (R)	25 (I)	13 (I)
10.1.3	*Ralstonia Eutropha*	R	15 (R)	R	19 (I)	R
10.1.4	*Pseudomonas nitroreducens/azelaica*	R	23 (S)	R	R	14 (I)
10.2.1	*Achromobacter xylosoxidans*	R	34 (S)	R	R	12 (I)
15.1.1	*Aeromonas media DNA group 5B*	19 (I)	27 (S)	21 (I)	29 (S)	14 (I)
15.1.2	*Pseudomonas nitroreducens/azelaica*	R	40 (S)	R	R	10 (R)
15.2.1	*Achromobacter xylosoxidans*	R	28 (S)	R	R	14 (I)
15.2.2	*Achromobacter xylosoxidans*	R	18 (I)	9 (R)	18 (R)	R
20.1.1	*Achromobacter xylosoxidans*	R	18 (I)	R	14 (R)	R
20.2.1	*Achromobacter xylosoxidans*	R	20 (I)	10 (R)	R	R
20.2.2	*Achromobacter xylosoxidans*	11 (R)	19 (I)	R	12 (R)	R
20.2.3	*Aeromonas caviae DNA group 4*	17 (I)	22 (S)	R	R	13 (I)

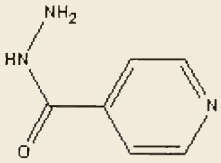
*( ) letters indicate the following: R = resistant, I = intermediate susceptibility, S = susceptible. Values of resistance were defined by BBL™ Sensi-Disc™ standards.

†The identification of the strains follows the procedure and criteria established by Biolog MicroLog1 4.20.

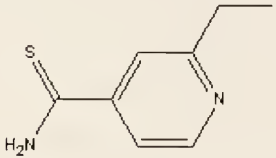
Figure 1. Structures of tested compounds and antibiotics.



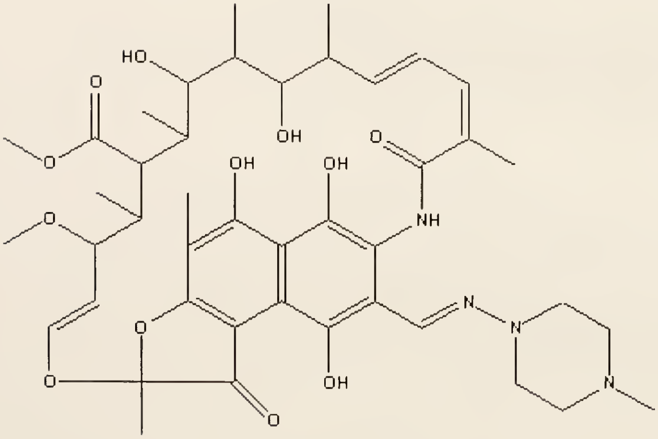
a. Triclosan



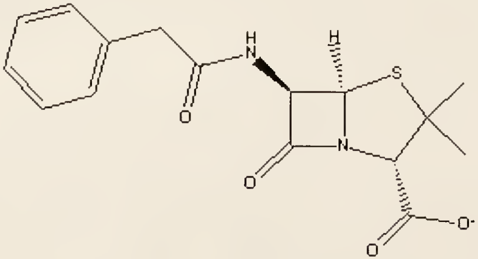
b. Isoniazid



c. Ethionamide

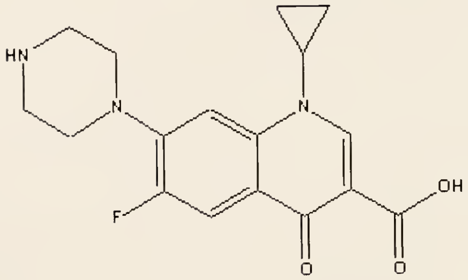


d. Rifampin

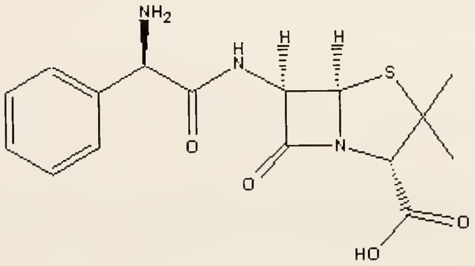


e. Penicillin

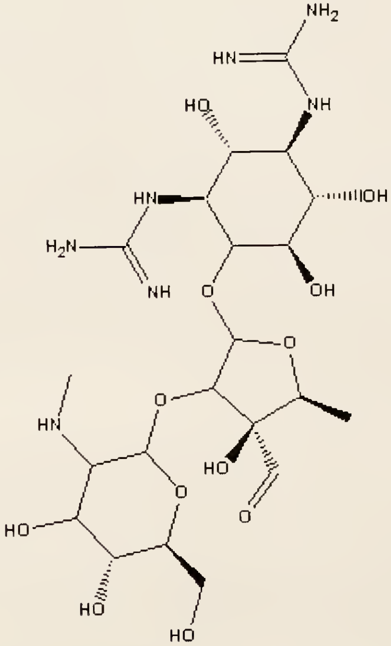
Figure 1. Continued.



f. Ciprofloxacin



g. Ampicillin



h. Streptomycin

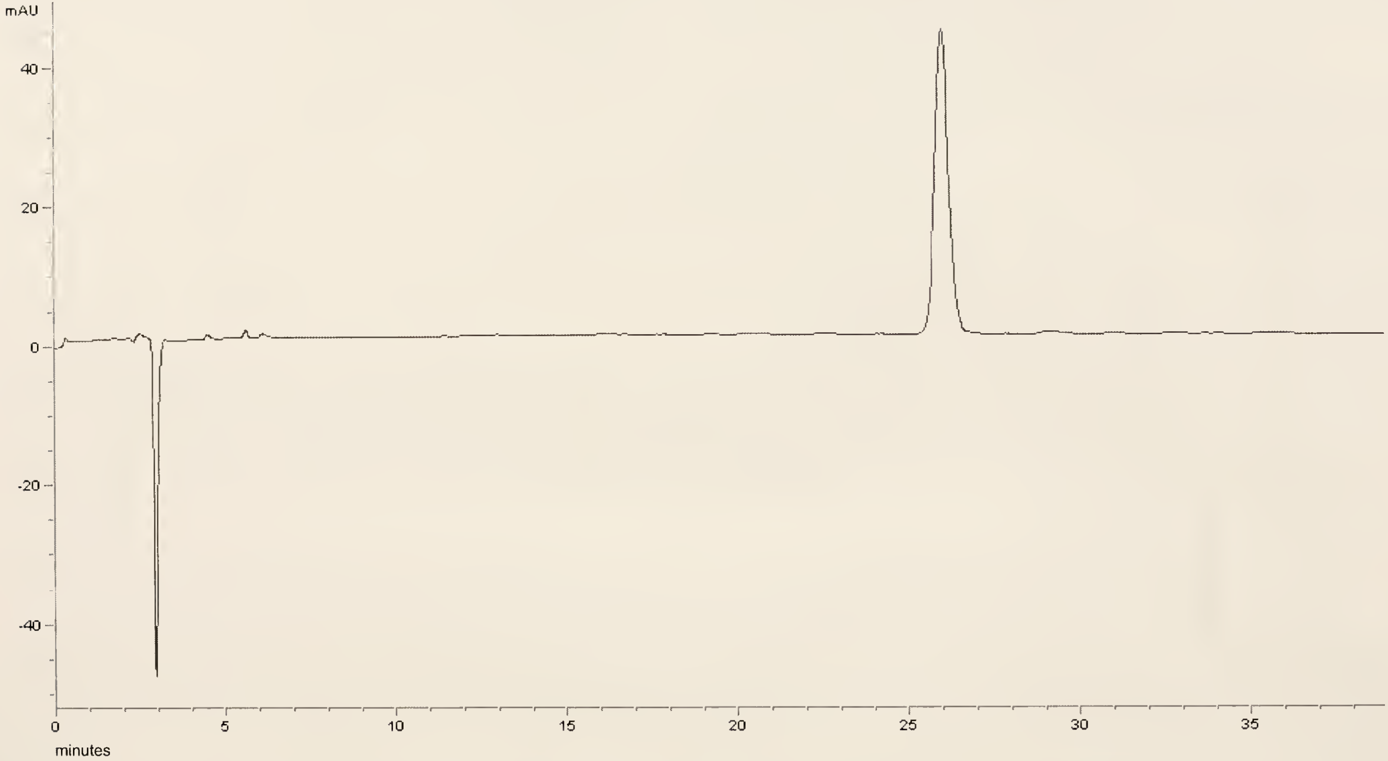


Figure 2. HPLC chromatogram of triclosan standard. The retention time of triclosan is 25.96 minutes.

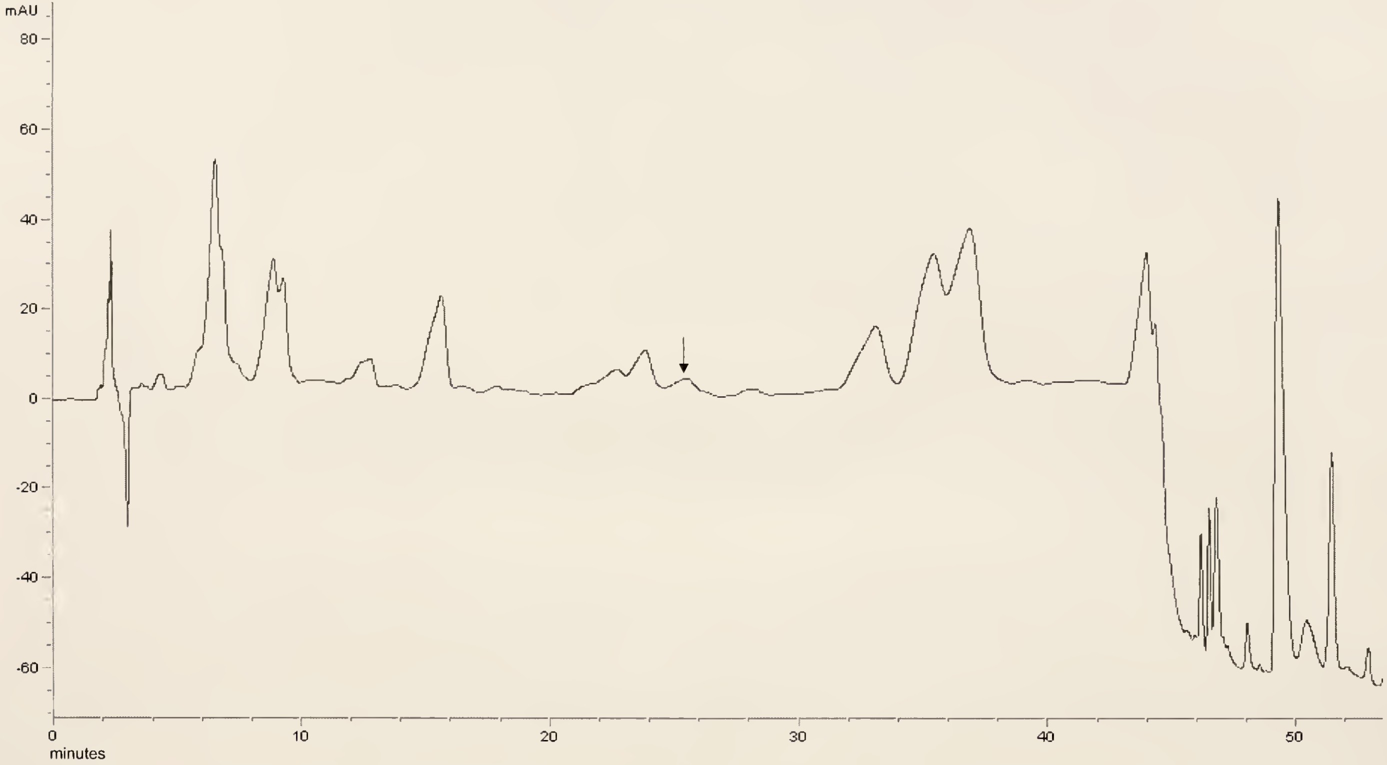


Figure 3. HPLC chromatogram of extracted primary influent from the Rivanna Wastewater Treatment Plant at Moores Creek in Charlottesville, VA. The retention time of triclosan is 25.47 minutes.

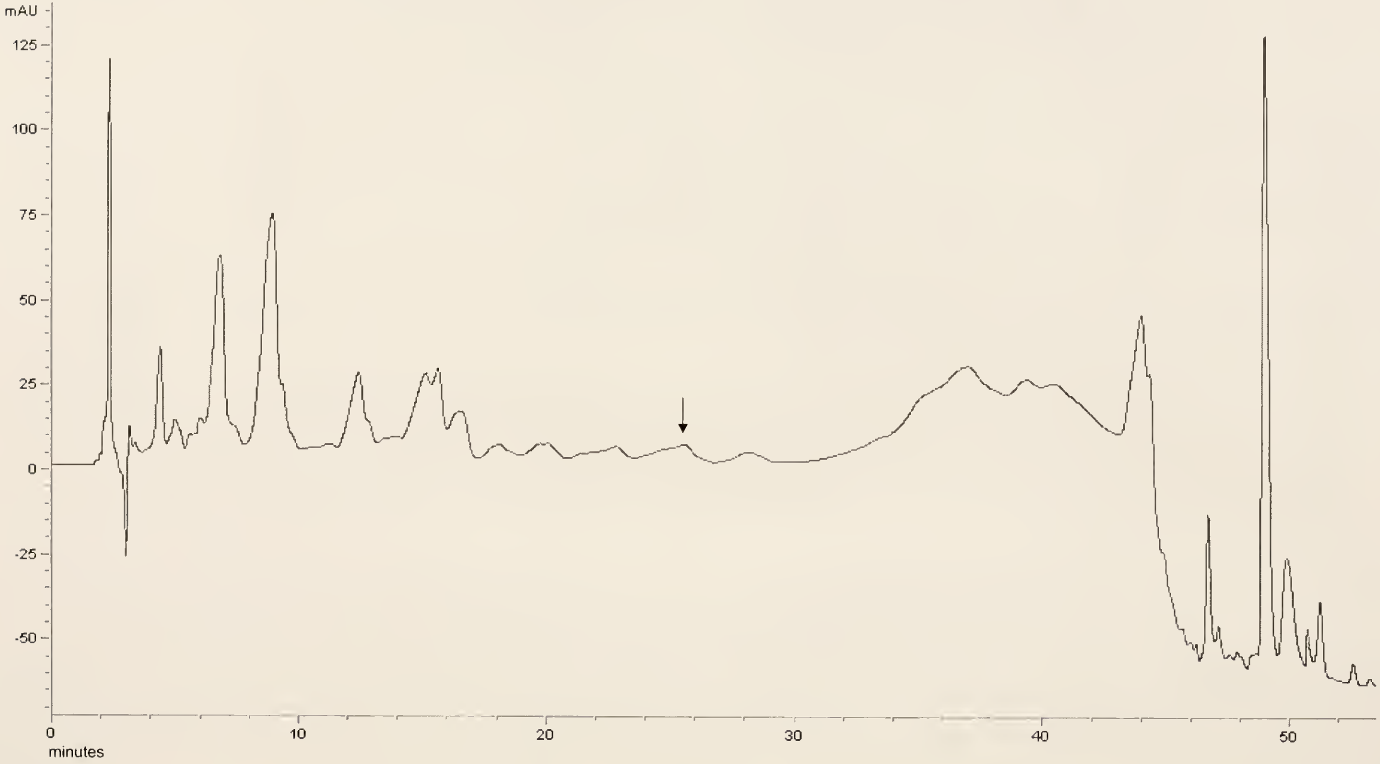


Figure 4. HPLC chromatogram of extracted primary influent from the Lexington Wastewater Plant in Lexington, VA. The retention time of triclosan is 25.49 minutes.

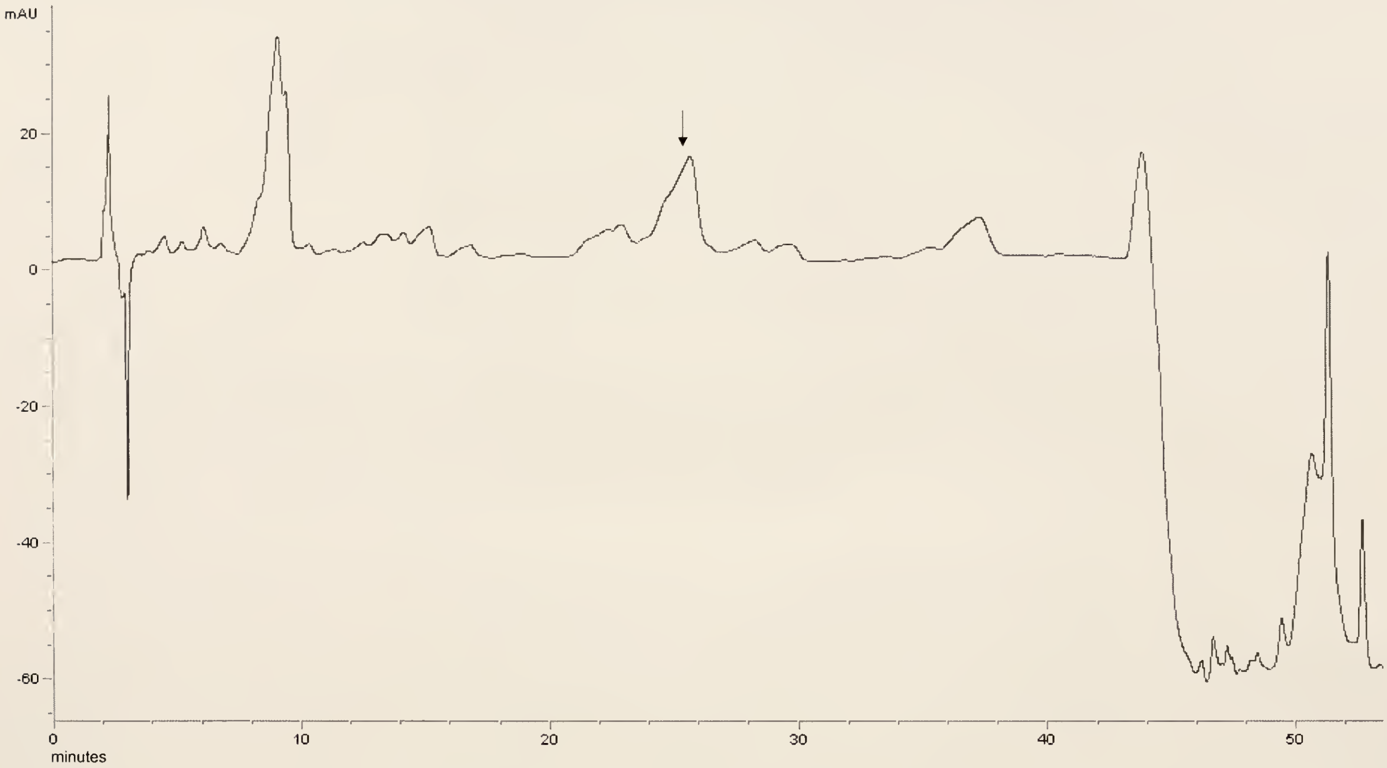


Figure 5. HPLC chromatogram of extracted primary influent from the Lynchburg Regional Wastewater Treatment Facility in Lynchburg, VA. The retention time of triclosan is 25.64 minutes.

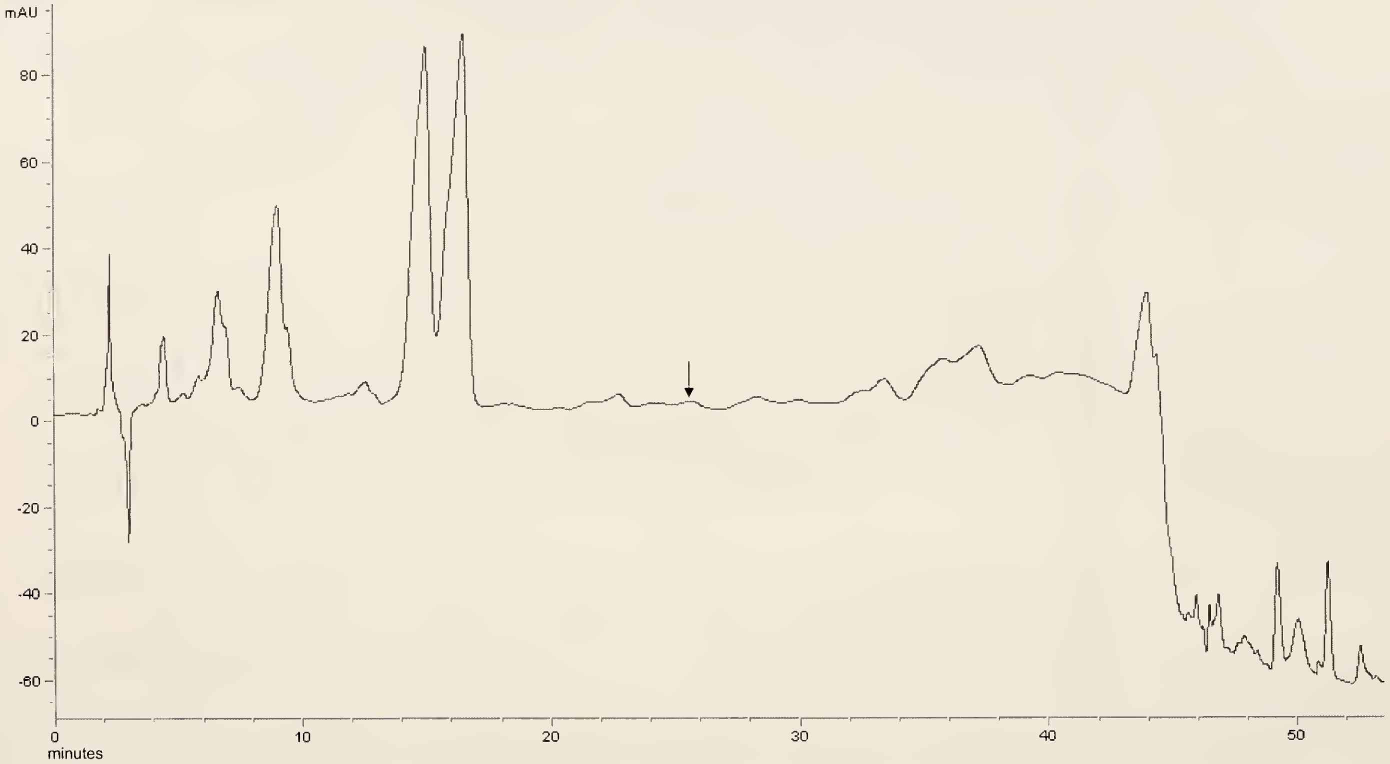


Figure 6. HPLC chromatogram of extracted primary influent from the Roanoke City Wastewater Treatment Plant in Roanoke, VA. The retention time of triclosan is 25.64 minutes.

Figure 7. Gas chromatogram of a 974-mg/l triclosan standard. Triclosan elutes from the capillary column at 19.33 minutes.

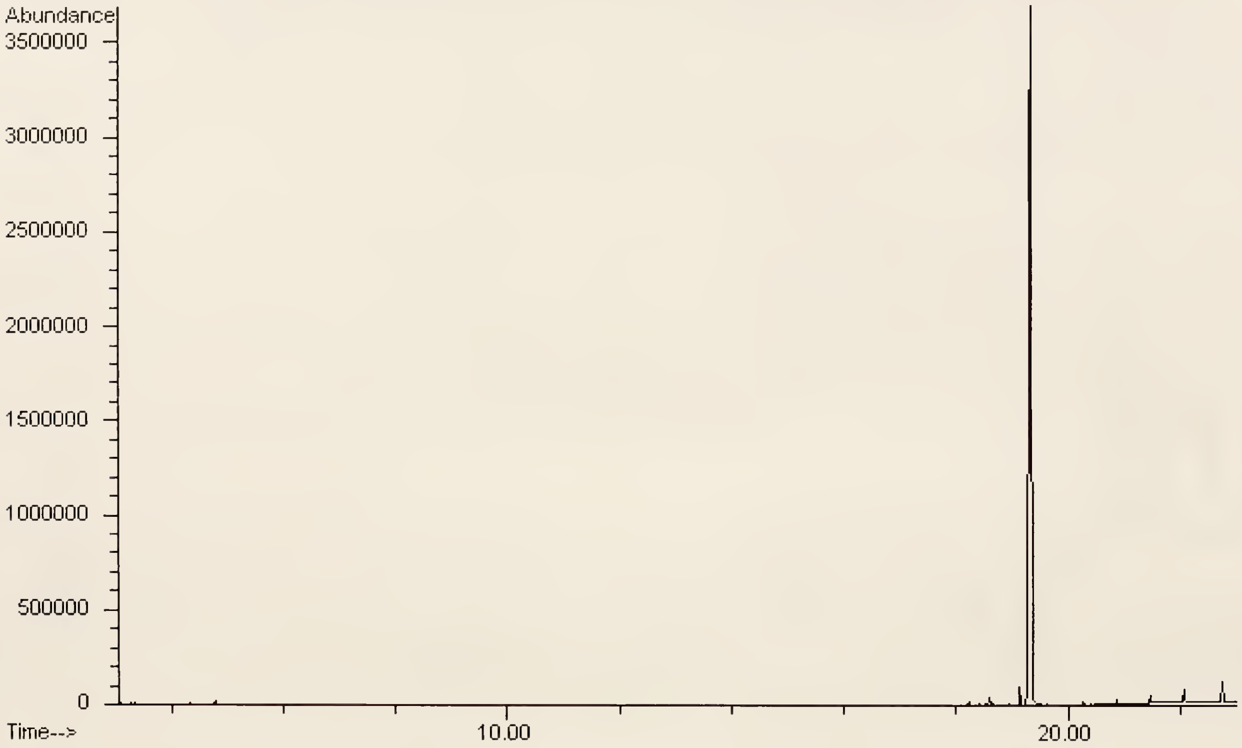


Figure 8. Gas chromatogram of an influent wastewater sample. This influent wastewater sample was collected from the Lynchburg Regional Wastewater Treatment Facility. Triclosan eluted from the capillary column at 19.275 minutes.

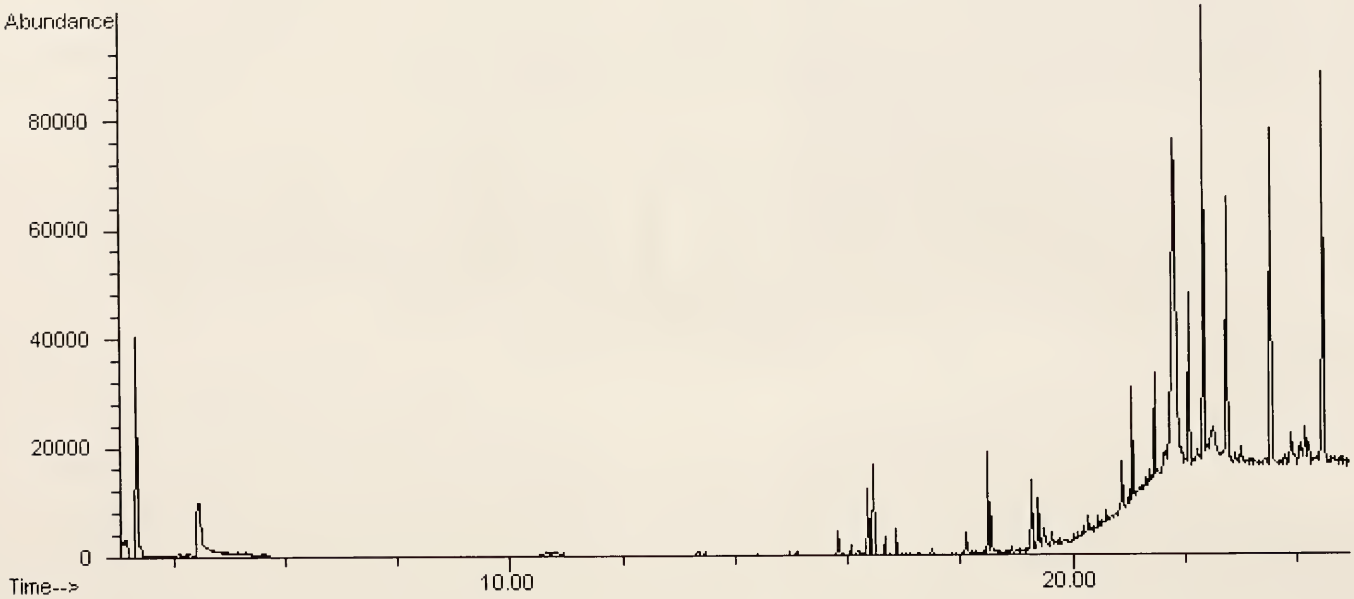


Figure 9. Mass spectra from a triclosan standard and a wastewater sample. Spectrum **(a)** is the spectrum taken from the injection of a triclosan standard at a retention time of 19.33 minutes. Similarly, spectrum **(b)** is the spectrum of an influent wastewater sample taken from the Lynchburg Regional Wastewater Treatment Facility. Both spectra have the expected triclosan fragmentation pattern.

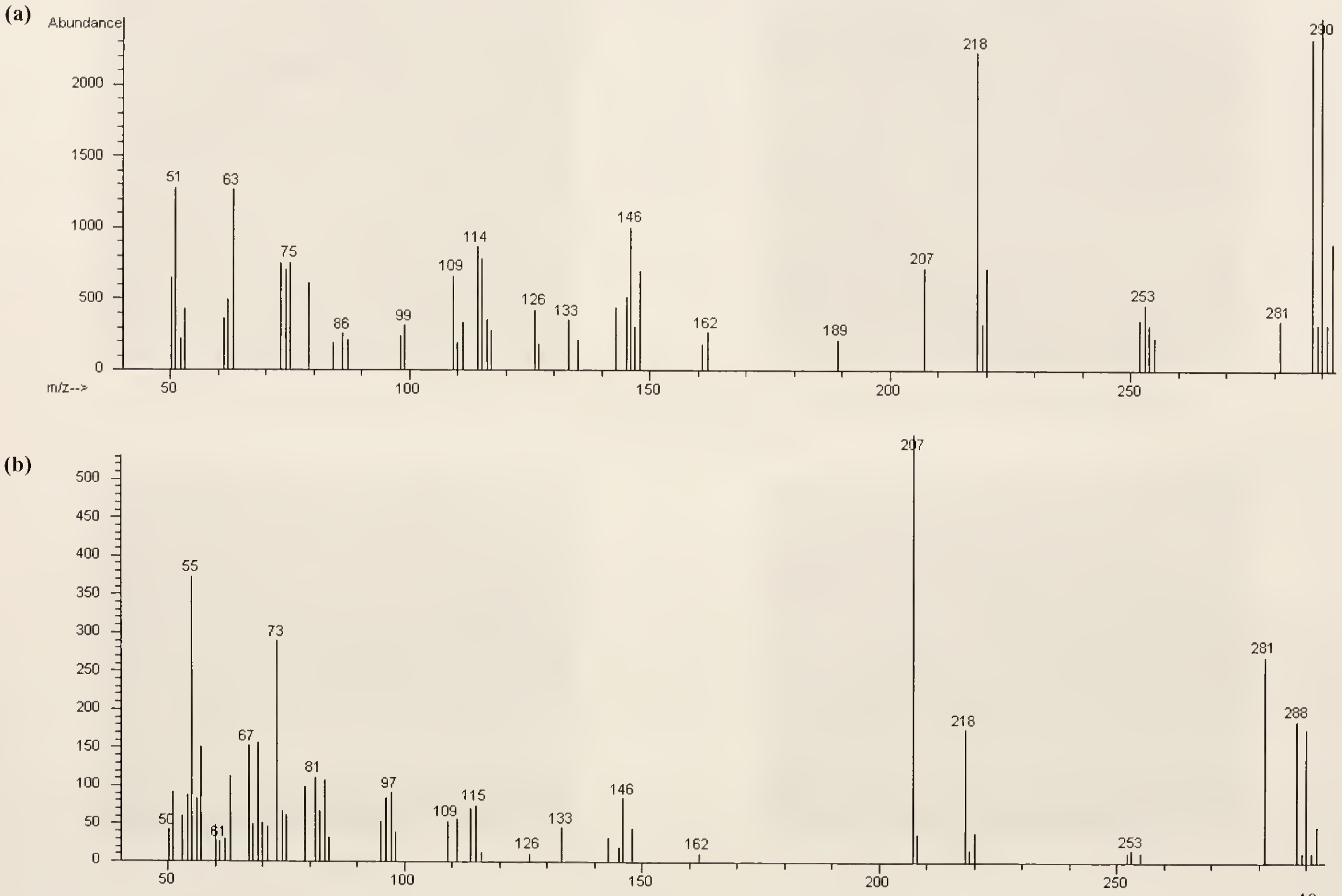
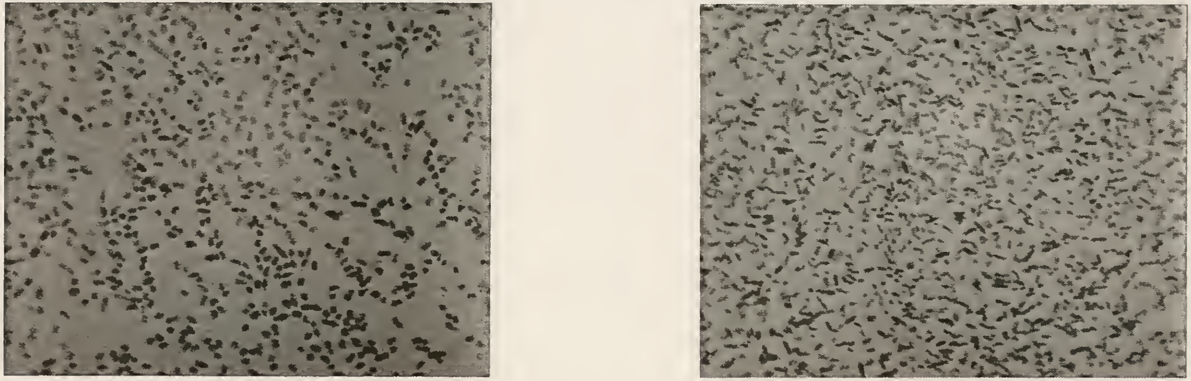
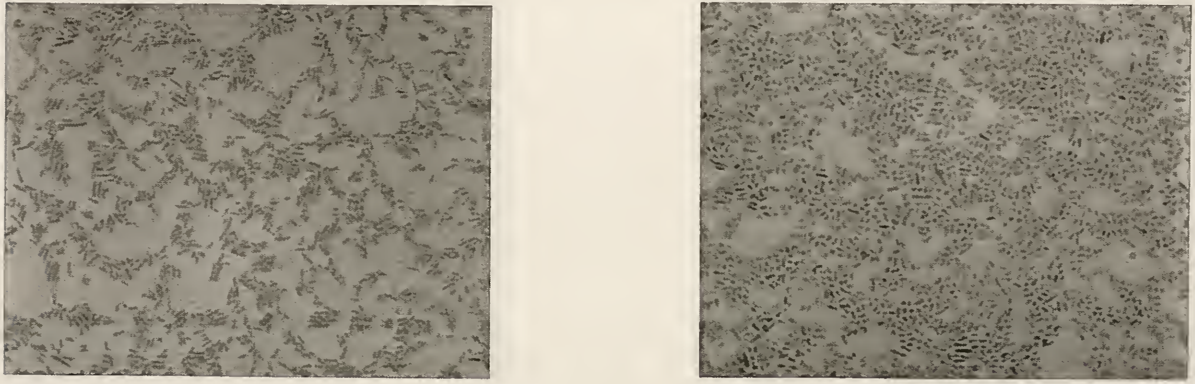


Figure 10. Micrographs of the triclosan-resistant organisms isolated from wastewater. Represented species include *Aeromonas*, *Ralstonia*, *Pseudomonas*, and *Achromobacter*.



Aeromonas caviae DNA group 4 (10.1.1)

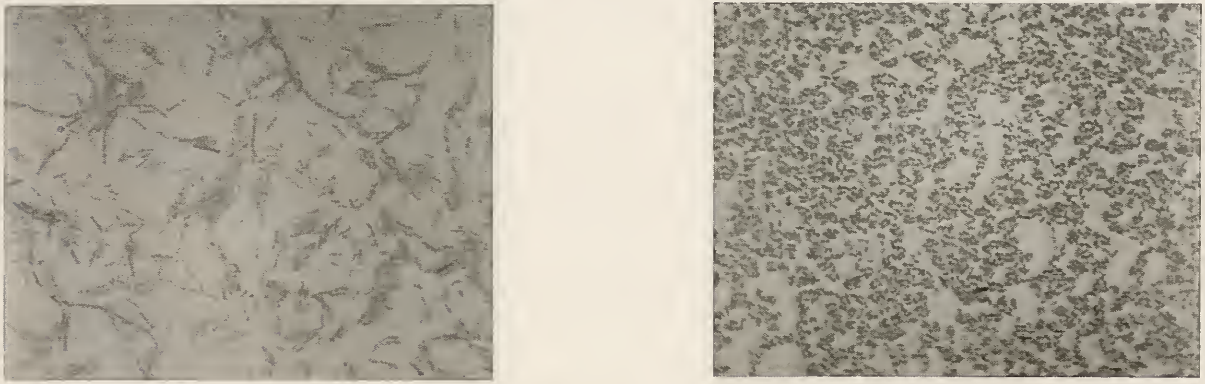
Aeromonas caviae DNA group 4 (10.1.2)



Ralstonia eutropha (10.1.3)

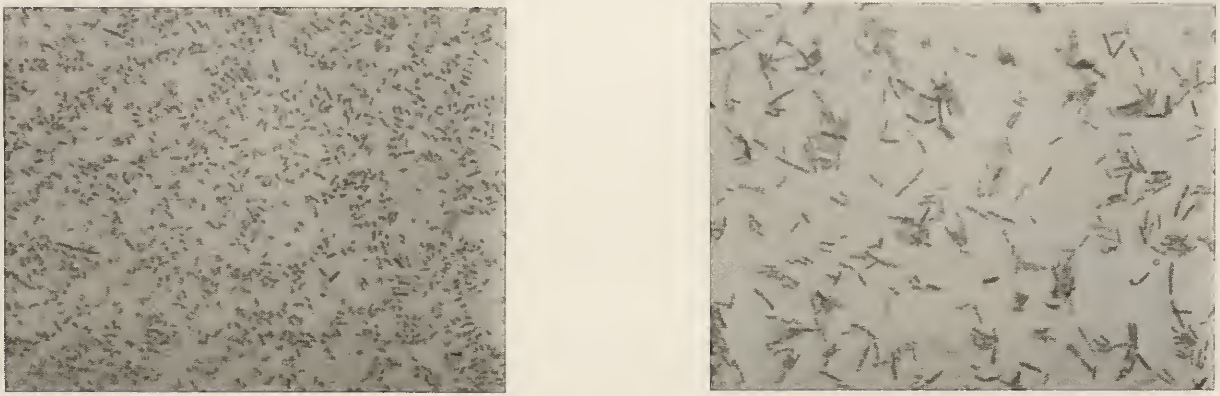
Pseudomonas nitroreducens/azelaica (10.1.4)

Figure 10. Continued.



Achromobacter xylosoxidans (10.2.1)

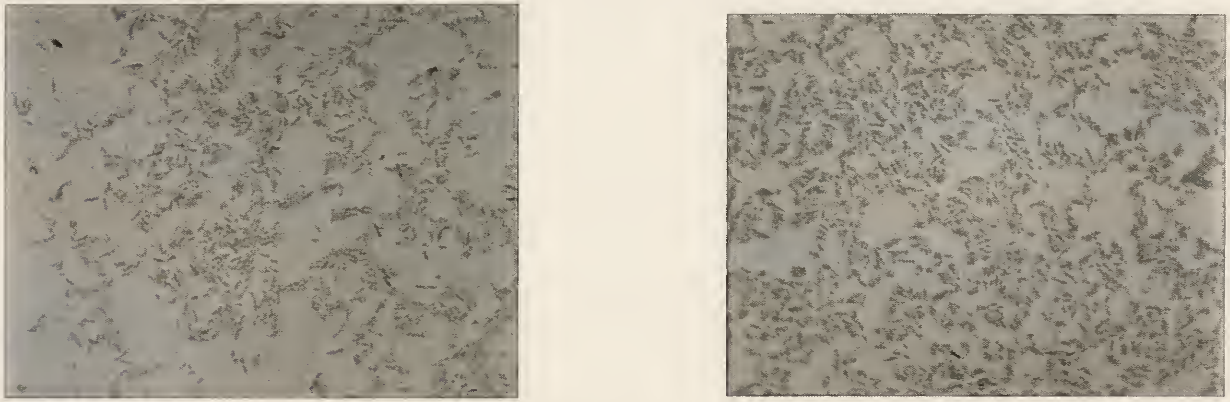
Aeromonas media DNA group 5B (15.1.1)



Pseudomonas nitroreducens/azelaica (15.1.2)

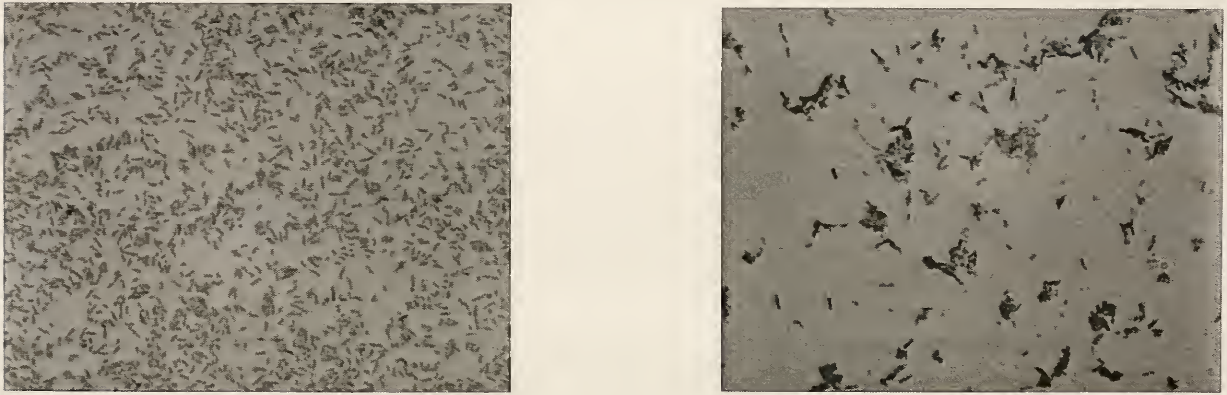
Achromobacter xylosoxidans (15.2.1)

Figure 10. Continued.



Achromobacter xylosoxidans (15.2.2)

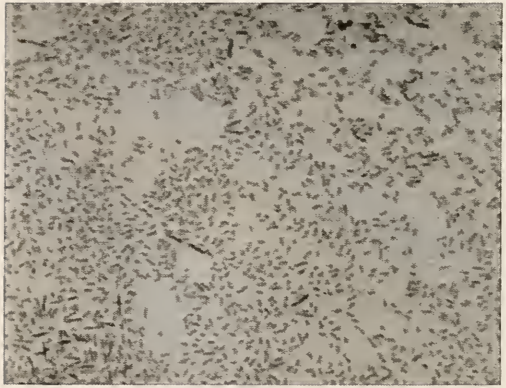
Achromobacter xylosoxidans (20.1.1)



Achromobacter xylosoxidans (20.2.1)

Achromobacter xylosoxidans (20.2.2)

Figure 10. Continued.



Aeromonas caviae DNA group 4 (20.2.3)

3282 2638 1
07/30/02 MAB